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Metastasis-suppressor genes suppress the growth of metastases without affecting tumor growth. We have been studying the role of inactivation of one such metastasis suppressor gene, Map Kinase Kinase 4 (MKK4) in the process of metastatic colonization. Work proposed in this application is aimed at extending our ongoing studies in the AT6.1 model system into new transgenic models of prostate cancer. In the past year we have made significant progress in the immunohistochemical studies proposed in the initial application and the development of gene targeting vectors for animal studies.

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## Table of Contents

## 1. Introduction

There is currently an upsurge in interest in cancer metastasis, the process by which malignant disease spreads throughout the body. Metastasis is a dynamic process beginning with dissemination of cells from the primary tumor followed by lodging and or extravasation of cells at a secondary site, and finally survival of cells at metastatic target sites. Despite improvements in the detection and treatment of primary tumors, metastatic disease remains refractory to current therapeutic interventions. Thus, presence of detecable metastases remains the hallmark of incurable disease.

Our work began more than ten years ago with the hypothesis that identification of genes that encode proteins which specifically suppress metastasis in vivo could lead to the identification of both biological and biochemical pathways that control specific steps of metastasis. These metastasis suppressor genes (MSGs) are operationally defined as genes that encode proteins that suppress the formation of overt metastases while exerting no measurable effect on primary tumor growth. Since then a wealth of data has been generated that shows that in addition to alterations in oncogenes and tumor suppressor genes, the acquisition of metastatic ability requires additional genetic and epigenetic changes. Functional studies as well as clinical correlative studies have identified metastasis suppressor functions for both novel and known genes (proteins) including NM23, MKK4, Brms1, Kiss1 and KAI1 and RKIP.

## 2. Body

We have previously identified a role for the stress-activated protein kinase (SAPK) signal transduction pathway in the suppression of metastatic colonization. Specifically, our laboratory identified the mitogen-activated protein kinase kinase 4/c-Jun NH2-terminal kinase-activating kinase/stress-activated protein/Erk kinase 1 (*MKK4/JNKK1/SEK1*, hereafter referred to as *MKK4*), as a metastasis-suppressor gene encoded by human chromosome 17p11.2 [1]. Ectopic expression of MKK4 in highly metastatic Dunning AT6.1 rat prostatic cancer cells suppressed the metastatic ability of the cells by approximately 77%. Additional studies showed that MKK4 metastasis-suppressed cells complete all early steps in the metastatic cascade, but are growth-inhibited at the secondary site. Kim *et al.* found that MKK4 expression was down regulated in clinical prostatic tumors with increased metastatic potential. In addition, a recent study by Yamada *et al.* demonstrated that MKK4 expression is

down regulated in ovarian cancer metastases. These findings support a role for MKK4 as a metastasis suppressor gene in clinical cancers.

Work proposed in this DOD Idea Award is aimed at extending these studies in to additional prostate cancer models. The two overall objectives are correlative studies of MKK4 expression in transgenic models of prostate cancer and to test the ability of MKK4 down regulation to promote metastasis in transgenic models of prostate cancer. Based on our previous studies we hypothesize the expression of MKK4 protein will be down regulated in high grade primary tumors that give rise to metastases and metastatic lesions.

## 3. Key Research Accomplishments

- We have developed optimized, consistent protocols for the sensitive and specific immunohistochemical (IH) detection of MKK4 in both paraffin embedded and frozen tissues.
- We have conducted a preliminary IH study of normal mouse prostates and primary TRAMP tumors.
- We have constructed a MKK4 targeting vector.

## 4. Reportable Outcomes

At this time our reportable outcomes are the techniques and tools that we have developed for protein detection and gene targeting. We have included details of these accomplishments since this work is not yet published.

- **We have developed optimized, consistent protocols for the sensitive and specific immunohistochemical (IH) detection of MKK4 in both paraffin embedded and frozen tissues.** *In order to perform studies to assess MKK4 protein expression we first had to develop a protocol for the consistent, sensitive and accurate immunohistochemical detection of MKK4. While this might appear to be a simple matter, the fact is that development of well-controlled, meaningful immunohistochemical studies is difficult.*

**Antibody Staining Protocol for Frozen TRAMP Tissues.** Tissue was embedded in O.C.T. (Tissue-Tek 4583) and snap frozen with liquid nitrogen. Five micron sections were cut and mounted on poly-l-lysine coated slides and air dried in fume hood overnight. Sections were frozen at -80°C until use. For Day 1 staining, sections were placed in 1X PBS at 4°C overnight. On Day 2, they were fixed in a series of organic solvents at 4°C. Organic solvent fixation involved 0.2% picric acid/2% w/v paraformaldehyde (Sigma P6744-1GA/ Polysciences 00380) in 0.1 M phosphate buffer (Fisher S369-500) pH 7.3 for 10'. Tissue sections were washed with 1X PBS for 5', then fixed in 85% EtOH/1.5% polyvinyl pyrrolidone (PVP) (Sigma P5288) for 5'. They were then washed in 1X PBS/1.5% PVP for 5'. Tissue aldehydes were blocked with 0.05% sodium borohydride in 1X PBS/1.5% PVP for 5'and then washed in 1X PBS/1.5% PVP for 5'. They were incubated in 1X PBS/1.5% PVP/0.1% Gelatin (Sigma G2625) for 5' followed by three 1X PBS washes.

Non-specific antigens were blocked with a 5% solution of normal goat serum (Vector)/0.5% casein (Sigma) in 1X PBS for 10 minutes at RT. Endogenous avidin and biotin was quenched by using the Vector avidin biotin blocking kit followed by three washes with the washing solution which contained 0.5% casein and 0.01% Tween 20 in 1X PBS.

Rabbit-anti-human, rat, mouse MEK-4 antibody (Santa Cruz (sc-964)) was titered to 1μg/ml and the Rabbit IgG isotype control (Santa Cruz (sc-2027)) was used at the same concentration for an antibody negative control. Other control slides run were MEK-4 antibody plus MEK-4 antibody blocking peptide (sc-964P), secondary antibody only, ABC only and DAB only. All antibodies were diluted in the antibody diluting solution containing 0.5% casein in 1X PBS. All slides were incubated at 4°C overnight.

For Day 3, the tissues were washed three times with the washing solution. Santa Cruz Goat-anti-rabbit IgG biotinylated, mouse/human absorbed, (sc-2040) secondary antibody was titered to 1μg/ml and incubated 25 minutes at 37°C and followed by three washes with the washing solution.

Endogenous peroxidase was quenched by incubating slides in 3% $H_2O_2$ (Fisher H325-500) in 1X PBS for 20 minutes at RT. The tissues were washed three times in 1X PBS. ABC Vectastain Standard Elite (PK-6100) was applied for 25 minutes at RT. The tissues were washed three times in 1X PBS, developed with the Vector DAB kit (SK-4100), and washed 1 X in distilled water.

Each was counterstained with the working solution (0.03%) of light green SF yellowish (Fisher 03382-25)) for 3-4 seconds and dehydrated through alcohols (10 dips in two changes each of 95% and 100% EtOH). Slides were dipped 10 times in two changes of xylene (X3P-1GAL) and mounted with permount (SP15-500) then coverslipped.

**Antibody Staining Protocol for Paraffin Embedded TRAMP Tissues**. Five-micron sections of paraffin embedded tissues were cut and mounted on poly-l-lysine coated slides. Sections were deparaffinized (xylene and alcohols) and antigens unmasked (Vector H-3300) for 15' in 95°C water bath and allowed to cool to RT. They were then fixed in a series of organic solvents at 4°C. Organic solvent fixation involved 0.2% picric acid/2% w/v paraformaldehyde (Sigma P6744-1GA/ Polysciences 00380) in 0.1 M phosphate buffer (Fisher S369-500) pH 7.3 for 10'. Tissues were washed with 1X PBS for 5', then fixed in 85% EtOH/1.5% polyvinyl pyrrolidone (PVP) (Sigma P5288) for 5'. They were then washed in 1X PBS/1.5% PVP for 5'. Tissue aldehydes were blocked with 0.05% sodium borohydride in 1X PBS/1.5% PVP for 5'and then washed in 1X PBS/1.5% PVP for 5'. They were incubated in 1X PBS/1.5% PVP/0.1% Gelatin (Sigma G2625) for 5'followed by three 1X PBS washes.

Non-specific antigens were blocked with a 5% solution of normal goat serum (Vector S-1000)/0.5% casein (Sigma C5890) in 1X PBS for 10 minutes at RT. Endogenous avidin and biotin was quenched by using the Vector avidin biotin blocking kit (SP-2001) followed by three washes with the washing solution which contained 0.5% casein and 0.01% Tween 20 in 1X PBS.

Rabbit-anti-human, rat, mouse MEK-4 antibody (Santa Cruz (sc-964)) was titered to 1μg/ml and the Rabbit IgG isotype control (Santa Cruz (sc-2027)) was used at the same concentration for an antibody negative control. Other control slides run were MEK-4 antibody plus MEK-4 antibody blocking peptide (sc-964P) and secondary antibody only. All antibodies were diluted

in the antibody diluting solution containing 0.5% casein in 1X PBS. All slides were incubated at 4°C overnight.

tissues were washed three times with the washing solution. Santa Cruz Goat-anti-rabbit IgG biotinylated, mouse/human absorbed, (sc-2040) secondary antibody was titered to 1μg/ml and incubated 25 minutes at 37°C and followed by three washes with the washing solution.

Endogenous peroxidase was quenched by incubating slides in 6% $H_2O_2$ (Fisher H325-500) in 100% methanol (Fisher A412-4) for 20 minutes at RT. The tissues were washed three times in 1X PBS. ABC Vectastain Standard Elite (PK-6100) was applied for 25 minutes at RT. The tissues were washed three times in 1X PBS, developed with the Vector DAB kit (SK-4100), and washed 1 X in distilled water.

Each was counterstained with the working solution (0.03%) of light green SF yellowish (Fisher 03382-25)) for 3-4 seconds and dehydrated through alcohols (10 dips in two changes each of 95% and 100% EtOH). Slides were dipped 10 times in two changes of xylene (X3P-1GAL) and mounted with permount (SP15-500) then coverslipped.

- **We have conducted a preliminary IH study of normal mouse prostates and primary TRAMP tumors.** We have completed staining of a set of normal and TRAMP prostates/tumors. An example of MKK4 immunostaining is shown in Figure 1. As can be seen the staining is specific for prostate epithelial cells. We are currently evaluating and scoring the staining on these samples.

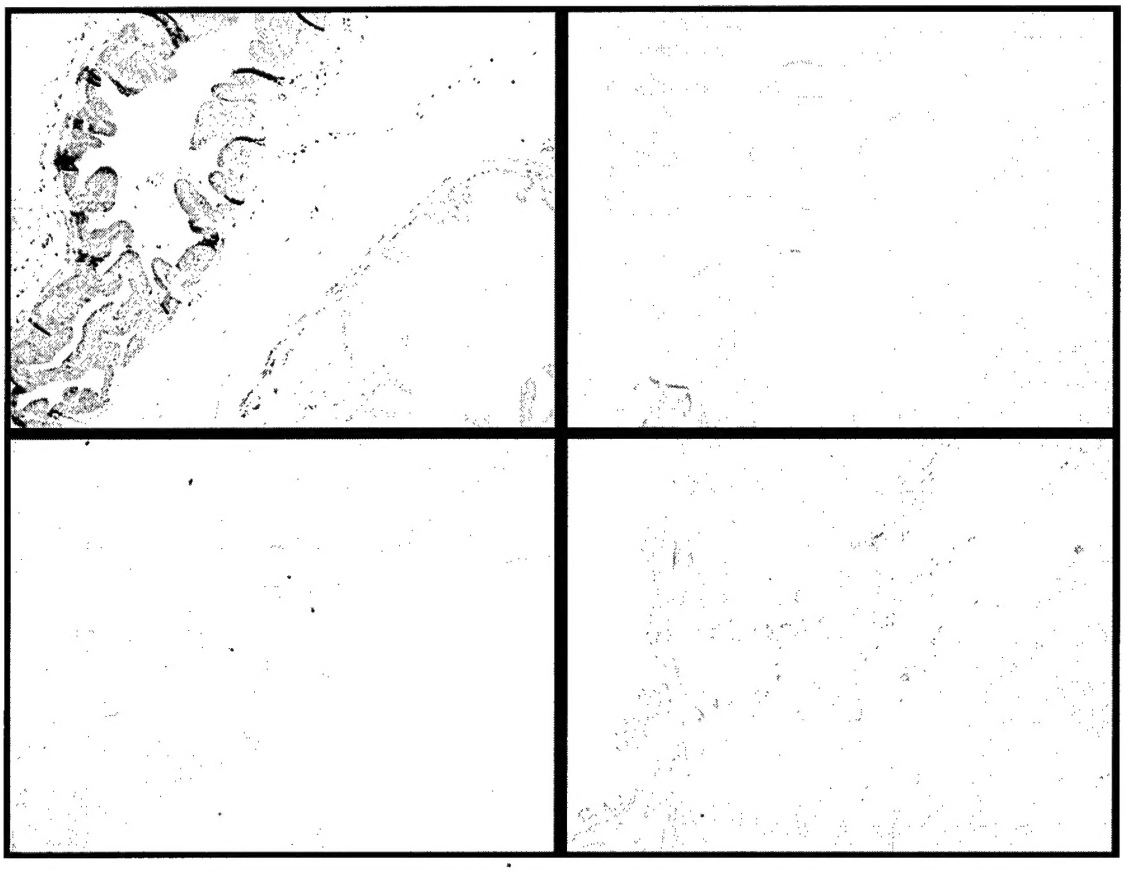

**Figure 1: MKK-4 staining of TRAMP Anterior Prostate 0002:68.** (A) MKK4 antibody 1ug/ml, (B) IgG isotype control, (C) secondary antibody only (D) Primary MKK4 antibody plus blocking peptide (peptide competition)

• **We have constructed a MKK4 targeting vector.** A mouse 129-embryonic stem cell phage library was plated (1 x $10^6$) and screened using a radiolabeled DNA probe corresponding to base pairs 772-1184 of the mouse MKK4 cDNA. Four possible clones resulted from the primary screen. After secondary and tertiary screens only one clone remained, which was subsequently purified and phage DNA was extracted. The genomic fragment was cut out using NotI and cloned into pBluescript (Stratagene). Restriction enzyme digests were done to generate a restriction map of the MKK4 genomic region using standard techniques (Current Protocols in Molecular Biology, Vol 1, 1994). Restriction mapping revealed that the 18.2 kb isolated genomic region contained MKK4/ SEK1 exons 7-11. The proposed targeting scheme requires the exon 6 and 7, therefore the isolated region was not usable.

An alternative targeting approach with several advantages was designed. In the new scheme, the entire genomic locus of MKK4/ SEK1 will be deleted by targeting upstream and downstream of the gene, as opposed to deleting exons 6 and 7 (the active region). In addition, the new scheme will allow for screening of embryonic stem cell clones *in vitro* to ensure that the appropriate homologous recombination events have taken place.

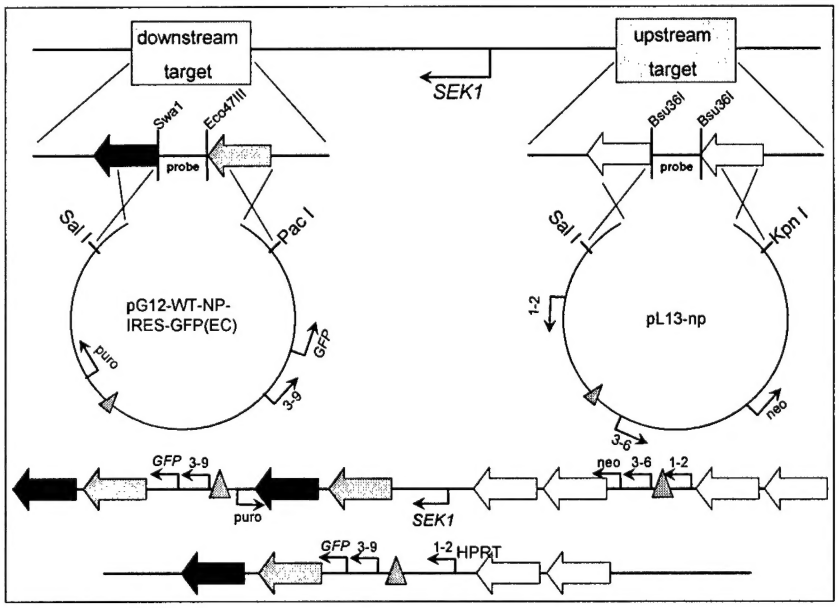


**Figure 2. Revised MKK4/SEK1 Targeting Scheme.**

Long and accurate (LA) PCR (Takara) was used to amplify 6kb regions of intronic sequence upstream and downstream of the MKK4/SEK1 gene. Amplified regions were selected by screening regions of Genbank mouse genomic DNA sequence upstream and downstream of MKK4/SEK1 for repetitive sequence using Repeat Masker (http://repeatmasker.genome.washington.edu). Primers were then designed and used to amplify the appropriate regions from mouse 129 genomic DNA. The resulting DNA product was cut with restriction enzymes (New England Biolabs). Targeting vector plasmids were cut with the corresponding restriction enzymes and the genomic fragments were ligated into the plasmids. MKK4/ SEK1 upstream was ligated into pL13-np and MKK4/SEK1 downstream was ligated into pG12-WT-NP-IRES-GFP (EC). Ligation products were transformed into E. coli (Invitrogen), and bacterial clones were screened using standard methods. A second restriction enzyme digestion and subsequent ligation was done for each plasmid to generate a region to be used for probe design to allow the identification of positive ES cell and mouse clones.

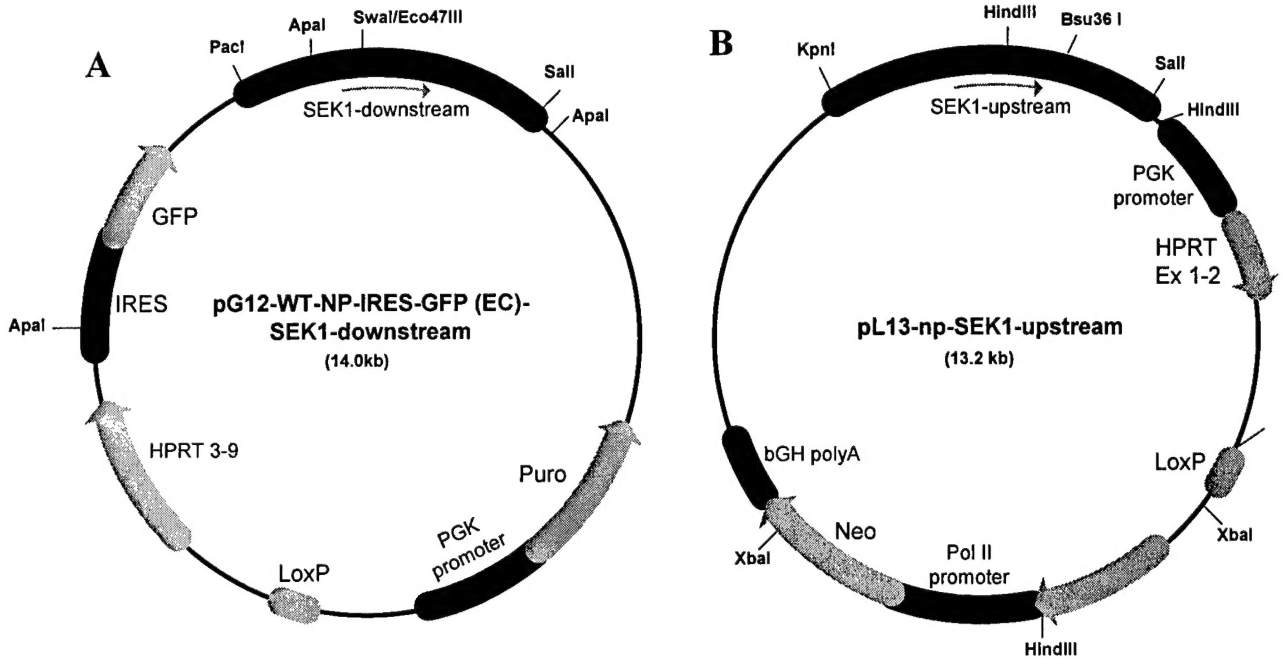


**Figure 3. Construction of Targeting Vectors.** ***Panel A*** Downstream Targeting Vector **Panel B** Upstream Targeting Vector

## 5. Conclusions

We now have the tools necessary to complete the IH evaluation of normal tissues, primary tumors and metastases from transgenic models of prostate cancer. We also have developed a targeting vector that we can begin to test for its utility in the proposed functional transgenic studies.

## 6. References

## 7. Appendices